

# APPENDIX 5

## Interaction of TAF<sub>II</sub>105 with Selected p65/RelA Dimers Is Associated with Activation of Subset of NF- $\kappa$ B Genes\*

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TAF<sub>II</sub>105, a substoichiometric coactivator subunit of TFIID, is important for activation of anti-apoptotic genes by NF- $\kappa$ B in response to the cytokine tumor necrosis factor (TNF)- $\alpha$ . In the present study we have analyzed the mechanism of TAF<sub>II</sub>105 function with respect to its regulation of p65/RelA, a component of NF- $\kappa$ B. We found two independent p65/RelA-binding domains within the N terminus of TAF<sub>II</sub>105. One of these domains appears to be crucial for TAF<sub>II</sub>105-mediated anti-apoptotic gene activation in response to TNF- $\alpha$ . Analysis of the interaction between TAF<sub>II</sub>105 and different NF- $\kappa$ B complexes has revealed substantial differences in the affinity of TAF<sub>II</sub>105 toward different p65/RelA-containing dimers. We have identified the TNF- $\alpha$  induced anti-apoptotic A20 gene as a target gene of TAF<sub>II</sub>105. A20 has a differential protective effect on cell death induced by TNF- $\alpha$  in the presence of either the dominant negative mutant of TAF<sub>II</sub>105 (TAF<sub>II</sub>105 $\Delta$ C) or the superdominant I $\kappa$ B $\alpha$ . The results suggest that the inhibitory effect of TAF<sub>II</sub>105 $\Delta$ C on NF- $\kappa$ B-dependent genes is restricted to a subset of anti-apoptotic genes while the effect of I $\kappa$ B $\alpha$  is more general. Thus, an interaction between NF- $\kappa$ B and a specific coactivator is important for specifying target gene activation.

An important issue in control of gene expression is the mechanism underlying the specificity of the cellular response to extracellular signals. In many cases this response is mediated by specific transcription factors that are activated by signaling pathways. In recent years considerable progress has been made in understanding the molecular mechanism by which signaling pathways regulate transcription in a variety of cellular systems. Nevertheless some serious problems remain unsolved. For instance, certain transcription factors can be activated by several distinct signals; yet, the genes induced by these factors and thus the cellular response vary significantly. The molecular basis for such differential activation of genes by specific transcription factors is not yet clear.

One family of transcription factors that is activated by a broad range of extracellular signals is NF- $\kappa$ B. The signals include inflammatory cytokines, chemokines, interferons, major histocompatibility complex proteins, growth factors, cell adhesion molecules, viruses, and certain stress signals (1).

NF- $\kappa$ B transcription factors also regulate apoptosis. They mediate activation of survival genes that protect cells from apoptosis induced by various agents such as the cytokine TNF- $\alpha$ <sup>1</sup> (2), certain oncogenes (3, 4), ionizing radiation, and chemotherapeutic agents (5). However, under some circumstances, NF- $\kappa$ B has the opposite effect, and can activate genes that promote apoptosis (6, 7).

The transcriptionally active NF- $\kappa$ B is a dimeric complex composed of members of the Rel family of proteins, p65/RelA, p50, c-Rel, RelB, and p52 (8). In most cells, these factors are localized in the cytoplasm bound by I $\kappa$ B proteins, a family of inhibitory proteins that prevents nuclear transport of NF- $\kappa$ B proteins. Signals that activate NF- $\kappa$ B, such as cytokines or stress, induce phosphorylation of I $\kappa$ B and its subsequent degradation, thereby releasing NF- $\kappa$ B. NF- $\kappa$ B then translocates into the nucleus and activates its target genes (1).

Previously we reported that hTAF<sub>II</sub>105 serves as coactivator for the p65/RelA NF- $\kappa$ B subunit and is required for activation of certain anti-apoptotic genes in human 293 cells (9). This function of hTAF<sub>II</sub>105 involves direct interaction between the p65 subunit of NF- $\kappa$ B and the N-terminal domain of hTAF<sub>II</sub>105. In addition to hTAF<sub>II</sub>105, numerous reports have indicated that the coactivator protein CBP and its homolog p300 are also involved in transcription activation by p65 subunit of NF- $\kappa$ B (10, 11). Likewise, other TFIID subunits such as hTAF<sub>II</sub>250, hTAF<sub>II</sub>80, and hTAF<sub>II</sub>28 have been reported to bind p65/RelA (12). Most recently, p65 was also found to interact specifically with the composite coactivator ARC/DRIP and this complex supports NF- $\kappa$ B dependent transcriptional activation *in vitro* (13). At present it is not clear whether these coactivator complexes display redundant functions, or whether transcription activation by NF- $\kappa$ B utilizes each of these complexes sequentially.

We report here a detailed analysis of TAF<sub>II</sub>105-p65/RelA physical and functional interaction. Our findings suggest that TAF<sub>II</sub>105 is involved in activation of only a subset of NF- $\kappa$ B genes and this selective effect of TAF<sub>II</sub>105 is associated with preference for selected forms of p65/RelA complexes. These results reveal the importance of combinatorial interactions between activators and coactivators for differential target gene activation.

### MATERIALS AND METHODS

**In Vitro Binding Experiments**—The N-terminal fragments of TAF<sub>II</sub>105 were expressed as GST fusion in *Escherichia coli*. The recombinant proteins were purified and immobilized on glutathione-Sepharose beads. <sup>35</sup>S-labeled p65 was synthesized *in vitro* by T7 RNA polymerase and rabbit reticulocytes lysate (Promega TNT kit), and incubated with the different GST purified proteins in 0.1 M KCl HEMG

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<sup>1</sup> The abbreviations used are: TNF, tumor necrosis factor; GST, glutathione S-transferase; RT-PCR, reverse transcriptase-polymerase chain reaction; CMV, cytomegalovirus; GFP, green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

buffer (20 mM HEPES, pH 7.9, 100 mM KCl, 12.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.1% Nonidet P-40, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride) for 2 h at 4 °C. The beads were washed 3 times with the same buffer and twice with 0.2 M KCl HEMG buffer. The bound proteins were eluted by boiling in protein sample buffer 5 min followed by SDS-PAGE and autoradiography.

Nuclear extracts (50  $\mu$ l, 15  $\mu$ g/ $\mu$ l) from BJAB cells in 0.1 M HEMG + 0.1% Nonidet P-40 were incubated with GST105 $\Delta$ C or with GST beads as described above. After the washes, bound proteins were eluted with 1 M NaCl HEMG, loaded on SDS-PAGE, and analyzed by Western blot, using anti-p65 and anti-p50 antibodies (Santa Cruz).

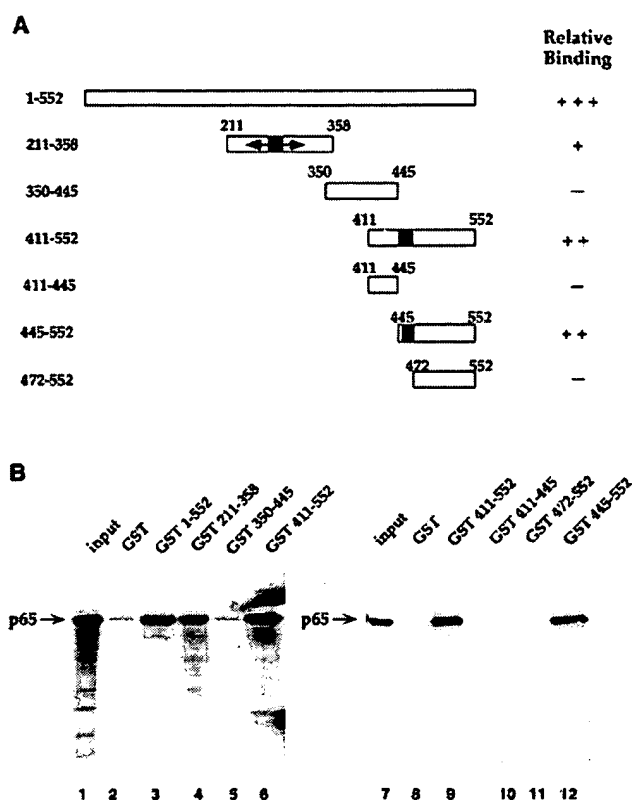
**Plasmids**—pCMV-TAF<sub>II</sub>105, pCMV-TAF<sub>II</sub>105 $\Delta$ C-(1–552) and pCMV-TAF<sub>II</sub>105 $\Delta$ C-(1–452) were previously described (9). TAF<sub>II</sub>105 $\Delta$ C-(445–471) was constructed by ligating a PCR fragment encoding amino acid 472–552 to the 3' of TAF<sub>II</sub>105 $\Delta$ C-(1–452) digested with *Hind*III and filled in. GST-TAF<sub>II</sub>105 $\Delta$ C-(1–552) was described previously. GST-TAF<sub>II</sub>105-(211–358) was constructed by ligating a *Sau*3A-*Pvu*II fragment into *Bam*HI-*Sma*I of pGEX2TK. GST-TAF<sub>II</sub>105-(350–445) was constructed by ligating an *Hae*III fragment-(1048–1335) into *Stu*I site of pGEX2TK. The DNA encoding amino acids 411–552 was generated by PCR and cloned into the *Nco*I-*Eco*RI sites of pGEX2TK. GST-TAF<sub>II</sub>105-(411–445) was generated by digesting 411–552 with *Nco*I and *Hae*III and ligating into pGEX2TK digested with *Nco*I and *Stu*I. 445–552 is an *Hae*III-*Eco*RI fragment from GST-TAF<sub>II</sub>105-(1–552) cloned into *Stu*I-*Eco*RI of pGEX2TK. The fragment encoding amino acids 472–552 was generated by PCR and cloned into the *Sma*I-*Bam*HI digest of pGEX3. The cDNA encoding the A20 gene (2.4 kilobases) was obtained by PCR from human Daudi cDNA library. The 5' oligonucleotide was AGTCTTCTCCTCAGGCTTGTA; and the 3' oligonucleotide, CCCACTTCTTGAGGAGGTGA. To construct the A20 expression plasmid, CMV-A20, the cDNA was cloned in pCGN vector in *Sma*I site. The insert was sequenced and expression was verified by Western blot using anti-hemagglutinin antibody (data not shown). To generate the A20 luciferase reporter plasmid, a *Pst*I fragment from A20-CAT (a gift of Dr. Dixit, V), spanning the proximal promoter region, was cloned in the *Pst*I site of pLuc, a promoter-less reporter plasmid. The NF- $\kappa$ B deleted A20 promoter was generated by PCR.

**RNA Preparation and RT-PCR Analysis**—Total RNA was prepared from 293 cells, untreated or treated with TNF- $\alpha$  for 1–3 h, using Trizol reagent (Life Technologies, Inc.), according to the manufacturers instructions. To determine the effect of TAF<sub>II</sub>105 and I $\kappa$ B on A20 mRNA, subconfluent 293T cells in 100-mm plates were transfected with 3.5  $\mu$ g of the different expression plasmids in a total of 10  $\mu$ g of DNA. RNA preparations were treated with RQ1 DNase I (Promega) to avoid contamination of genomic and transfected DNAs. First strand cDNA and PCR amplification were performed in a single tube using the Access RT-PCR System (Promega). The oligonucleotides used for RT-PCR are: *GAPDH* forward, TTGTCATCAATGGAAATCCC; *GAPDH* reverse, TGTGCTGTGTAAGTCAG; A20 forward, CACACAAGGCATCTGGATCC; A20 reverse, CAGGATGTCTTGCAGGAGG; A1 forward, CAA-GACTTTGCTCTCCACA; A1 reverse, GGCAATCGTTTCCATATCA-GT; *MnSOD* forward, ATGTTACAGCCCAGACAGC; *MnSOD* reverse, TTCAATCACTTGCCCAATAA; *IEX-1L* forward, AGGCAACTTGAAC-TCAGAACAA; *IEX-1L* reverse, CTGCGAGCCACCCTAA; *Bcl-xL* forward, ACCCATCTGGCACCTGGCA; *Bcl-xL* reverse, GGATCCAAGGC-TCTAGGTGG; *cIAP2* forward, GAAATAAGGGAAGAGGAGAG; *cIAP2* reverse, TACGAAGTGTACCCCTTGATT. Oligonucleotides used as probes for the RT-PCR products were *GAPDH*, TGAAGCAGCGCTCG-GAGGGC, and A20, AGATCCCTCGCGGCTCGTCC.

**Propagation and Transfection of Cell Lines**—293 cells are embryonic kidney fibroblasts. 293T cells express SV40 large T-antigen and therefore allow replication of plasmids bearing SV40 origin. 293, 293T, and HT1080 cell lines were maintained in F-12 Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Transfections were performed using the standard Ca/PO<sub>4</sub> method.

For apoptosis assays, 293 cells were transfected in 6-well plates using a total of 3  $\mu$ g of plasmid DNA/transfection. Transfections were performed with 0.2–0.6  $\mu$ g of p65 DNA; 0.5–0.6  $\mu$ g of TAF<sub>II</sub>105 or TAF<sub>II</sub>105 $\Delta$ C DNA; 0.5–0.6  $\mu$ g of I $\kappa$ B DNA and 0.3–0.5  $\mu$ g of A20. Reporter assays were performed as described previously (9). The amount of the different activator plasmids varied between 5 and 20 ng, and TAF<sub>II</sub>105 plasmid used was 300–500 ng/well. In each transfection the amount of CMV containing plasmid was kept constant. Luciferase activity was determined according to the manufacturer's protocol (Promega).

**Electrophoretic Mobility Shift Assay**—Nuclear extracts were prepared as described (38) from 293 cells treated with 10 ng/ml TNF- $\alpha$  for 1 h, or 48 h after transfection of NF- $\kappa$ B subunits as indicated. A double stranded oligonucleotide, containing the NF- $\kappa$ B site was labeled with



**FIG. 1. Mapping of p65-binding domain on TAF<sub>II</sub>105.** A, schematic representation of TAF<sub>II</sub>105 deletion mutants derived from its N-terminal region. These mutants were analyzed for p65/RelA binding as shown in B. The relative binding was determined by normalizing p65 binding activity to the amount of recombinant protein used and is shown on the right as + or -. B, pull-down binding assay using <sup>35</sup>S-labeled p65/RelA and purified fragments of TAF<sub>II</sub>105 (indicated by their amino acid residues) fused to GST and bound to glutathione beads. As control, a similar reaction was performed using the same beads bound by GST (lanes 2 and 8). The input lanes represent 10% of p65 used for the binding assay.

Klenow fragment of DNA polymerase I as probe. The oligonucleotides sequence is: 5'-GATCCAGAGGGGACTTTTCCGAGAG-3'; 5'-GATC-CTCTCGGAAAGTCCCCTCTG-3'.

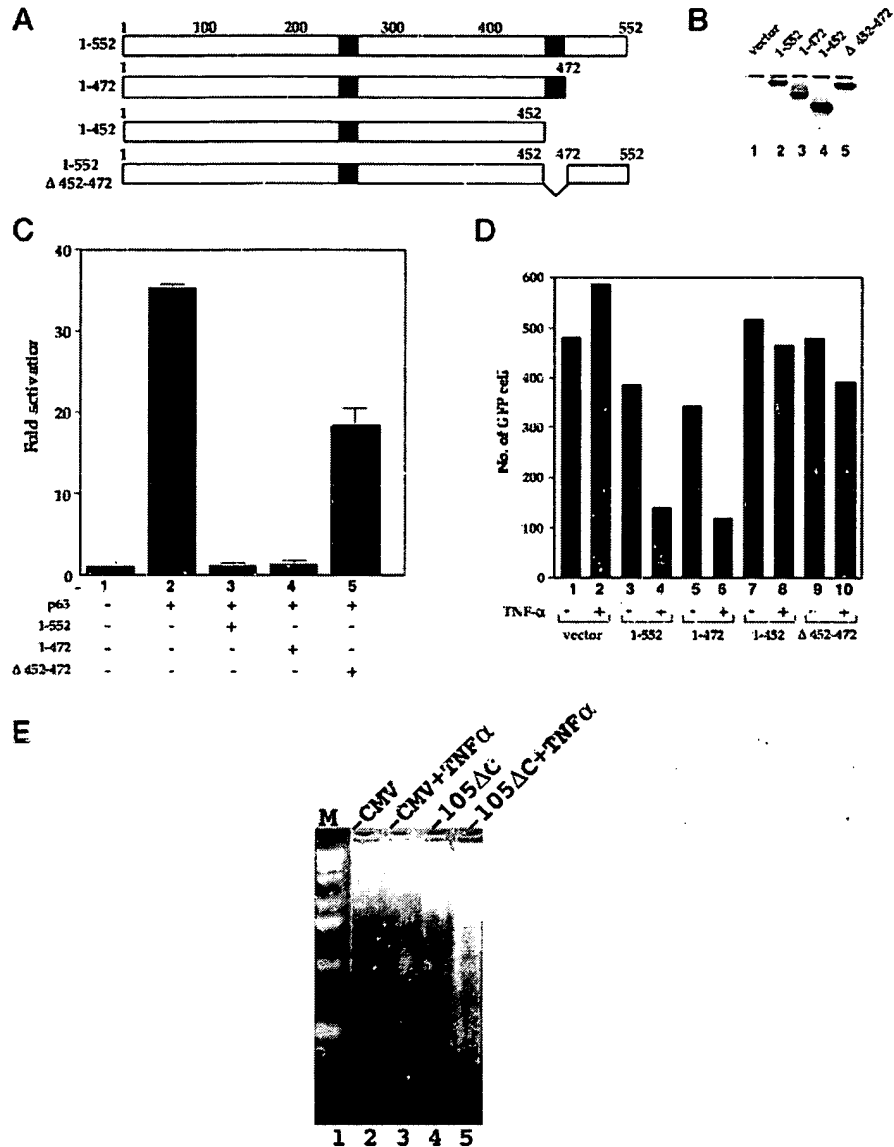
Purified recombinant GST or GST-TAF<sub>II</sub>105 fragments (1  $\mu$ g) were added to the binding reaction together with the DNA probe and incubated at room temperature for 30 min before loading onto the gel.

**Cell Survival Assay**—For the survival analysis 293 cells in 6-well plates were co-transfected with pBabe-GFP reporter plasmid (50–100 ng) together with the indicated expression vectors (500 ng each). TNF- $\alpha$  (5 ng/ml) was added 12–24 h after transfection and 48–60 h after transfection cells were visualized by microscope for green fluorescent cell detection. The number of GFP cells was determined by counting three different randomly chosen fields.

## RESULTS

**Inhibition of Anti-apoptotic Gene Activation by Dominant Negative Mutant of TAF<sub>II</sub>105 Involves Direct Interaction with p65/RelA**—TNF- $\alpha$  induction stimulates a protein synthesis independent apoptotic response. In cells resistant to the TNF- $\alpha$  cytotoxic effect, it also induces expression of proteins that block apoptosis and this activation is mediated by NF- $\kappa$ B. TAF<sub>II</sub>105 interacts with the p65/RelA subunit of NF- $\kappa$ B and is involved in activation of NF- $\kappa$ B-dependent anti-apoptotic genes in human 293 cells (9). To examine the mechanism of TAF<sub>II</sub>105 action with respect to NF- $\kappa$ B, we mapped the domain within the TAF<sub>II</sub>105 N terminus directing the interaction with p65/RelA. For this purpose various fragments of TAF<sub>II</sub>105 N-terminal domain were cloned in front of the glutathione S-transferase (GST) gene (Fig. 1A). The different fusion proteins were puri-

**FIG. 2. Functional analysis of TAF<sub>II</sub>105 $\Delta$ C mutants.** A, schematic representation of TAF<sub>II</sub>105 $\Delta$ C derived mutants cloned in mammalian expression plasmids. B, immunoblot of 293 cells transfected with the either empty vector (lane 1) or different TAF<sub>II</sub>105 $\Delta$ C mutants (lanes 2–5) using anti-hemagglutinin antibody. C, effect of TAF<sub>II</sub>105 $\Delta$ C mutants on p65-dependent transcription activation. Human 293 cells were co-transfected with an NF- $\kappa$ B-dependent luciferase reporter, together with an empty expression plasmid (column 1) or p65 (column 2), or p65 with different mutants of TAF<sub>II</sub>105 $\Delta$ C (columns 3–5). Luciferase activity was monitored 24 h post-transfection. D, survival analysis of cells transfected with the different TAF<sub>II</sub>105 $\Delta$ C mutants and induced by TNF- $\alpha$ . 293T cells were co-transfected with CMV-GFP reporter plasmid and an empty vector or the expression plasmids described in A. 24 h after transfection TNF- $\alpha$  was applied to the cells and 48 h later, fluorescent cells in five randomly chosen fields were counted. These data are representative of three independent transfection experiments with similar results. E, 293T cells were transfected with an empty expression plasmid (lanes 2 and 3) and TAF<sub>II</sub>105 $\Delta$ C (4 and 5) and either untreated (lanes 2 and 4) or treated with 15 ng/ml TNF- $\alpha$  (lanes 3 and 5). Transfection efficiency was estimated to be ~80%. Genomic DNA was extracted 48 h after transfection and analyzed by 1.8% agarose gel. M indicates DNA size marker (lane 1).

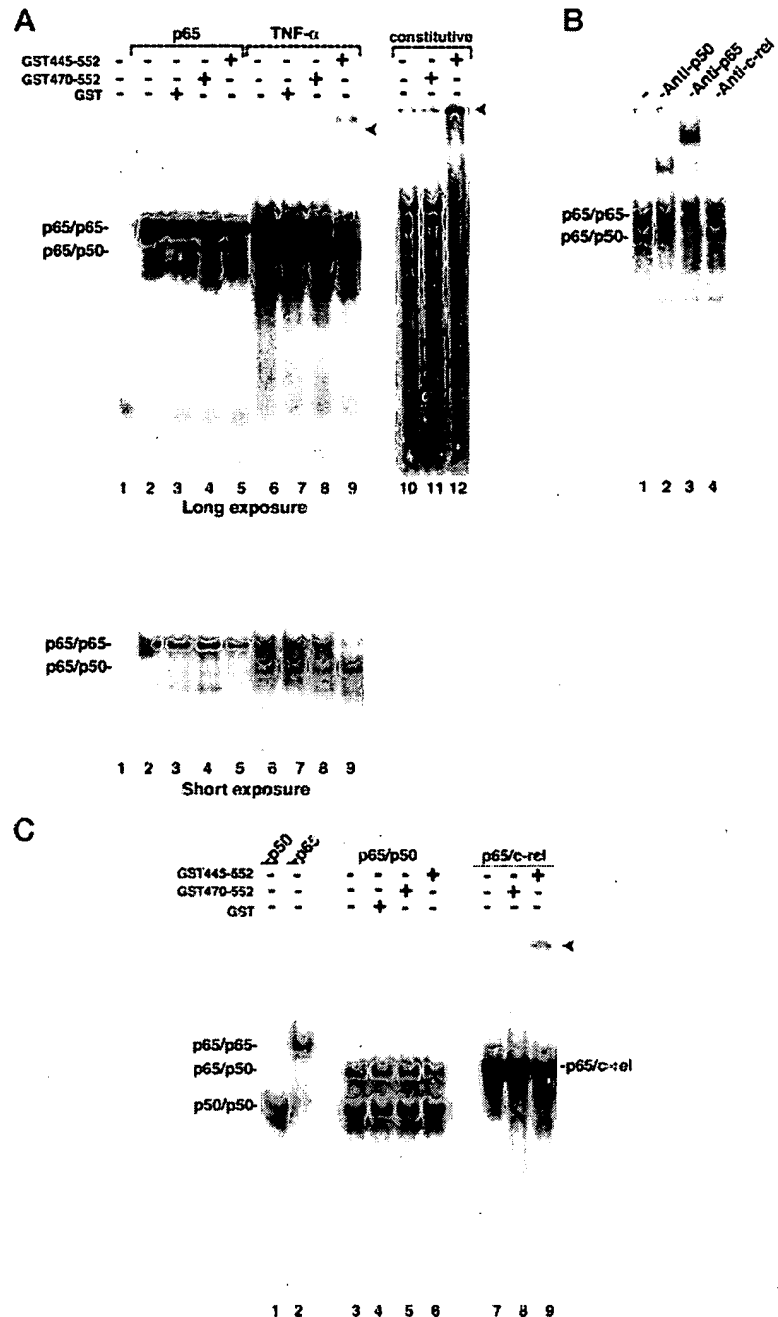


fied from *E. coli* and used for an *in vitro* pull-down assay with <sup>35</sup>S-labeled p65/RelA subunit of NF- $\kappa$ B (Fig. 1B). This analysis revealed two p65/RelA-binding regions within TAF<sub>II</sub>105 (Fig. 1B, lanes 4 and 6). Quantitation of the relative binding indicated that the C-terminal-binding domain interacts with p65/RelA more efficiently. Further deletion analysis of the major p65/RelA-binding site indicated that the amino acid residues between 445 and 472 are crucial for p65 binding (lanes 7–12).

Based on the binding experiments we constructed mammalian expression plasmids carrying mutated variants of TAF<sub>II</sub>105 $\Delta$ C, a dominant negative form of TAF<sub>II</sub>105 (Fig. 2A). We next analyzed the inhibitory effect of TAF<sub>II</sub>105 $\Delta$ C mutants on p65/RelA-mediated transcription activation in 293 cells. As shown in Fig. 2C, the TAF<sub>II</sub>105 $\Delta$ C mutant lacking the major p65-binding domain is a less potent inhibitor of p65/RelA transcription activity than the mutants carrying p65-binding domain (columns 3–5). Next, we examined the effect of these mutants on NF- $\kappa$ B-dependent anti-apoptotic gene activation in response to the cytokine TNF- $\alpha$ . We used the green fluorescent protein (GFP) expression assay to measure cell viability of transfected cells (Fig. 2D). A significant reduction in cell viability in response to TNF- $\alpha$  is observed in cells expressing any

form of TAF<sub>II</sub>105 $\Delta$ C containing the p65/RelA binding (Fig. 2D) as occurred also in cells expressing superdominant I $\kappa$ B (Fig. 7). We confirmed that this effect is related to programmed cell death by analyzing the genomic DNA of cells expressing TAF<sub>II</sub>105 $\Delta$ C and induced with TNF- $\alpha$  (Fig. 2E). The DNA ladder characteristic to apoptotic response is clearly observed in cells expressing TAF<sub>II</sub>105 $\Delta$ C and stimulated with TNF- $\alpha$  (lane 5) while no DNA fragmentation appeared in TNF- $\alpha$ -induced 293 cells transfected with empty expression vector (lane 3). Notably, the survival of 293 cells seems to be partially dependent on the constitutive nuclear NF- $\kappa$ B activity since expression of either superdominant I $\kappa$ B (Fig. 7) or TAF<sub>II</sub>105 $\Delta$ C (Fig. 2D) reduces by 20–30% cell survival of transfected cells without providing external apoptotic signal. TAF<sub>II</sub>105 $\Delta$ C lacking the p65/RelA-binding region failed to inhibit the basal as well as TNF- $\alpha$  induced anti-apoptotic gene activation and to sensitize cells to TNF- $\alpha$  cytotoxic effect (Fig. 2D, columns 7–10). These results suggest that the p65-binding domain of TAF<sub>II</sub>105 is crucial for its anti-apoptotic function.

**Differential Binding of Distinct Forms of NF- $\kappa$ B to TAF<sub>II</sub>105**—Transcription activation through the NF- $\kappa$ B regulatory site can be directed by different dimers of the NF- $\kappa$ B



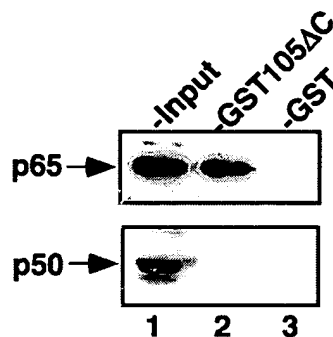
**FIG. 3. Differential binding of TAF<sub>II</sub>105 to different forms of NF- $\kappa$ B.** **A**, electrophoresis mobility shift assay using the NF- $\kappa$ B-binding site as DNA probe. Nuclear extracts were prepared from control (lanes 1 and 10–12 shown after longer exposure), p65 transfected (lanes 2–5), and 1 h TNF- $\alpha$ -treated 293 cells (lanes 6–9). Purified recombinant GST or GST fused to TAF<sub>II</sub>105 fragments were added to the DNA binding reactions as indicated at the top each lane. **B**, identification of NF- $\kappa$ B subunits of the TNF- $\alpha$  induced complexes using NF- $\kappa$ B specific antibodies as indicated. **C**, analysis of TAF<sub>II</sub>105 binding by transfected p50/p50, p65/p50, and p65/c-Rel complexes. Antibodies against p65 and c-Rel were used to confirm the presence p65 and c-Rel in the cell extract prepared from p65 + c-Rel-transfected cells (data not shown).

family. p65/RelA can dimerize with all the family members. A possible regulatory mechanism for specifying gene transcription would be if various combinations of NF- $\kappa$ B dimers display differential coactivator binding. We have compared the relative binding of TAF<sub>II</sub>105 to different forms of NF- $\kappa$ B complexes: constitutive, TNF- $\alpha$  induced, and transfected NF- $\kappa$ B dimers. Nuclear extracts, prepared from control, TNF- $\alpha$ -induced, and NF- $\kappa$ B-transfected 293 cells, were used in electrophoresis mobility shift assays with a labeled oligonucleotide specific to the NF- $\kappa$ B-binding site. The DNA binding reaction was incubated with TAF<sub>II</sub>105 recombinant fragment which included the major p65-binding domain (GST-(445–551)). A mutated TAF<sub>II</sub>105 protein lacking the p65-binding region (GST-(470–551)) was used as a control (Fig. 3). As expected, strong NF- $\kappa$ B binding activity was detected following TNF- $\alpha$  induction or transfection of various NF- $\kappa$ B members (Fig. 3, A and C). TNF- $\alpha$  induced

the formation of two major NF- $\kappa$ B binding complexes (Fig. 3A). Using antibodies directed against different subunits of the NF- $\kappa$ B family, we identified the low mobility complex as a homodimer of p65 and the faster migrating complex as a p65/p50 subunit heterodimer (Fig. 3B). Interestingly, among the TNF- $\alpha$  induced NF- $\kappa$ B complexes, the p65/p65 homodimer but not the p65/p50 complex formed a ternary supershifted complex with the wild type although not with the p65-binding site-deleted TAF<sub>II</sub>105 peptide (Fig. 3A, lanes 6–9). The formation of the supershifted complex is accompanied with 53% reduction in the intensity of p65/p65 complex and less significant reduction in the intensity of p65/p50 complex (5%) as determined by densitometric analysis (see also Fig. 3A, short exposure). Similarly, the constitutive NF- $\kappa$ B complex which is also composed of a p65 homodimer (data not shown), bound TAF<sub>II</sub>105 efficiently (Fig. 3A, lanes 10–12). Incubation of p65

homodimeric complex derived from transfected cells with the GST-(445–552) TAF<sub>II</sub>105 fragment resulted in a weaker p65 complex (Fig. 3A, lane 5, 38% reduction). A weak ternary supershifted complex could be seen only after prolonged exposure (data not shown). It is likely that this ternary complex is not stable under the electrophoresis conditions indicating this type of p65 homodimeric complex bound TAF<sub>II</sub>105 less efficiently than native p65/p65 complex such as the TNF- $\alpha$  induced or the constitutive p65/p65 complex (Fig. 3A compare lanes 5–9 and 12). This suggests that the affinity of TAF<sub>II</sub>105 for the p65 homodimer may also be regulated, perhaps by signaling pathways. p65/p50 or p50/p50 dimers derived from transfected cells were impaired in TAF<sub>II</sub>105 association in this assay (Fig. 3C, lanes 3–6). In contrast, the p65/c-Rel heterodimer did bind to TAF<sub>II</sub>105 (Fig. 3C, lanes 7–9).

To further test the association of TAF<sub>II</sub>105 with NF- $\kappa$ B proteins we used nuclear extract prepared from the B cell line BJAB. This extract was incubated with immobilized TAF<sub>II</sub>105 (N terminus, amino acid 1–552) and bound NF- $\kappa$ B subunits were analyzed by Western blot using antibodies specific to



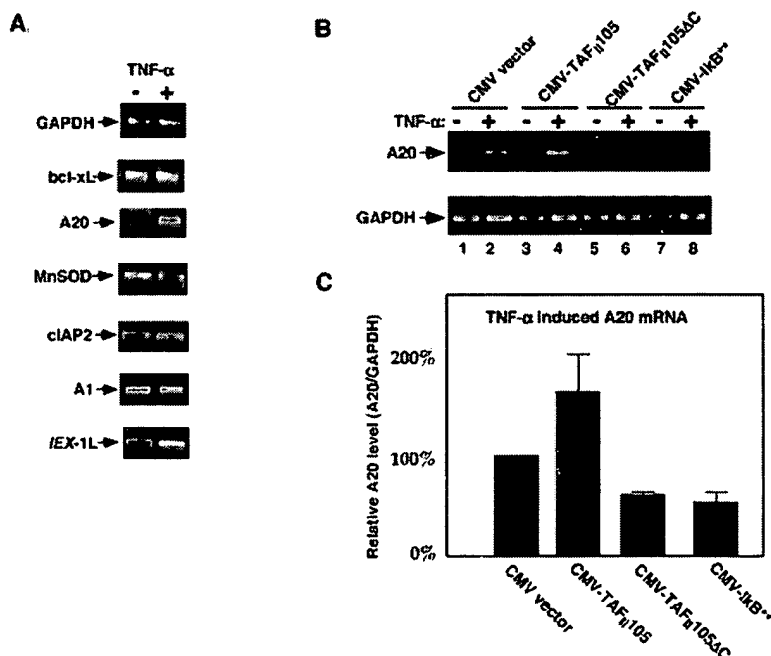
**FIG. 4. Selective interaction of hTAF<sub>II</sub>105 with p65 homodimer.** Purified TAF<sub>II</sub>105 protein (GST105ΔC, amino acid 1–552) fused to GST and bound to glutathione-Sepharose beads, or control GST-containing beads were used for binding reaction with nuclear extract prepared from the human B cell line BJAB. The bound proteins were eluted by high salt and subjected to SDS-polyacrylamide gel electrophoresis followed by Western blot analysis using anti-p65 or anti-p50 antibodies. Input lanes represent 10% of the total nuclear extract used for the binding.

either p65 or p50 subunits of NF- $\kappa$ B. As shown in Fig. 4, only p65 but not p50 was specifically retained on TAF<sub>II</sub>105 containing beads. Since this BJAB extract contains p65/p50 complex (data not shown), this experiment further confirms the preference of TAF<sub>II</sub>105 to p65 dimers devoid of the p50 subunit. Taken together these findings indicate that NF- $\kappa$ B dimers vary in their affinity to TAF<sub>II</sub>105.

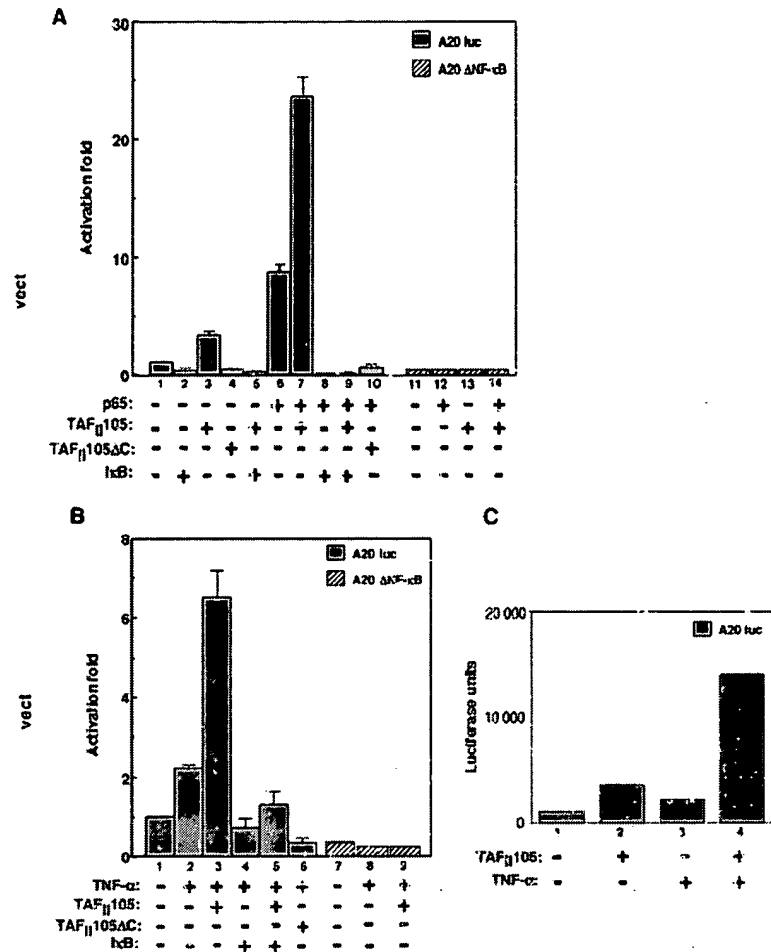
**The TNF- $\alpha$  Inducible Anti-apoptotic Gene A20 Is a Transcriptional Target of TAF<sub>II</sub>105**—Numerous studies have indicated that NF- $\kappa$ B anti-apoptosis involves activation of several genes that inhibit, partially or fully, the TNF- $\alpha$  cell death response. In an attempt to identify anti-apoptotic gene(s) targeted by TAF<sub>II</sub>105 in 293 cells, we first examined the expression of these genes in response to short term TNF- $\alpha$  induction using RT-PCR. We analyzed the manganese superoxide dismutase gene (14), the A20 gene (15), cIAP2, a member of inhibitor of apoptosis family of genes (16, 17), the bcl-2 homolog A1 gene (18) and IEX-1L (19). We also tested the expression of BclX<sub>L</sub>, an anti-apoptotic gene that is not induced by TNF- $\alpha$  and the housekeeping gene GAPDH. Of all these genes, the A20 gene was rapidly and most significantly induced by TNF- $\alpha$  (Fig. 5A). The IEX-1L was moderately induced and the rest of the genes analyzed were not induced at all by TNF- $\alpha$ . The finding that only two out of the five NF- $\kappa$ B-dependent anti-apoptotic genes that we analyzed are TNF- $\alpha$  inducible in 293 cells suggests that in different cell types, resistance to TNF- $\alpha$  induced cell death is conferred by different sets of anti-apoptotic genes.

To examine the possible involvement of TAF<sub>II</sub>105 in A20 transcription, we tested whether the endogenous A20 mRNA level is affected by TAF<sub>II</sub>105 expression. 293 cells were transiently transfected with wild type or dominant negative mutant TAF<sub>II</sub>105ΔC and treated with TNF- $\alpha$  for 1 h. RNA was extracted and used to monitor the level of the A20 mRNA by RT-PCR reaction. TAF<sub>II</sub>105 expression elevates the TNF- $\alpha$  induced A20 mRNA whereas TAF<sub>II</sub>105ΔC inhibits it (Fig. 5, B and C). The effect of TAF<sub>II</sub>105 is specific since it had no significant effect on the level of GAPDH mRNA. In agreement with a previous study (20), we found that the transcription of the A20 gene is NF- $\kappa$ B dependent, since expression of superdominant I $\kappa$ B similarly inhibited A20 transcription. Thus, induction A20 mRNA by TNF- $\alpha$  involves NF- $\kappa$ B proteins and the TFIID

**FIG. 5. A, analysis of TNF- $\alpha$  inducible genes in human 293 cells.** 293 cells were treated with TNF- $\alpha$  (5 ng/ml) for 1 h and expression of the indicated genes was determined by RT-PCR. These results are representative of two to four experiments with similar results. **B, effect of TAF<sub>II</sub>105 expression on mRNA level of the TNF- $\alpha$  induced A20 gene.** Human 293T cells were transfected with the indicated expression plasmids (transfection efficiency is above 50%). 24 h post-transfection cells were treated with TNF- $\alpha$  for 1 h followed by RNA extraction and RT-PCR reaction using human A20 or GAPDH specific primers. The identity of the PCR products was confirmed by southern hybridization (data not shown). **C, quantitation of A20 RT-PCR product level normalized to GAPDH products.** These results are average of three independent transfection experiments with similar results.



**FIG. 6. The effect of TAF<sub>II</sub>105 on the A20 promoter activity.** A, human 293 cells (in 24-well dish) were co-transfected with wild type or NF- $\kappa$ B-deleted A20-dependent reporter plasmid (25 ng) together with either empty expression vector or the following expression plasmids: TAF<sub>II</sub>105 (150 ng, columns 3, 5, 7, 9, 13, and 14), p65 (3–5 ng, columns 6–10, 12, and 14), 500 ng of I $\kappa$ B $\alpha$  (columns 2, 5, 8, and 9), and 500 ng of TAF<sub>II</sub>105 $\Delta$ C (columns 4 and 10). The amount of CMV derived vector in each transfection assay was kept constant. B, similar transfection experiment using TNF- $\alpha$  instead of p65. 24 h after transfection TNF- $\alpha$  (1.5–3 ng/ml) was added and 3 h later cells were harvested. C, similar transfection assays as in B using HT1080 cells. The results presented are average of three independent transfection assays with similar results.



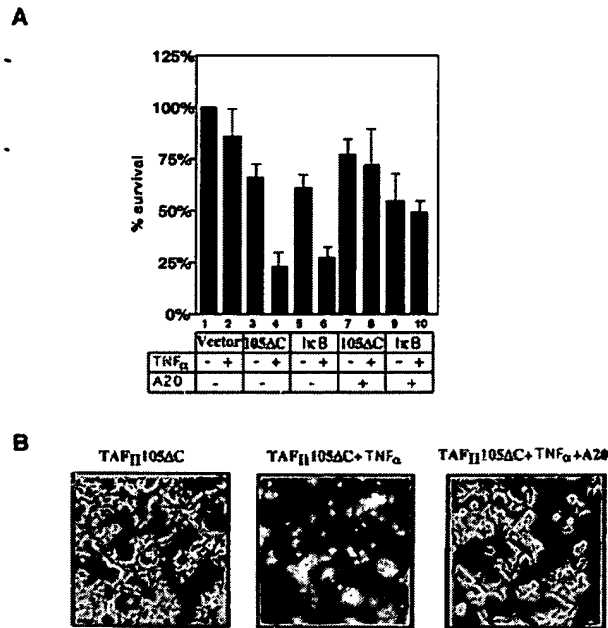
complex containing TAF<sub>II</sub>105.

**TAF<sub>II</sub>105 Modulates A20 Promoter Activity**—The proximal promoter region of the A20 gene contains two NF- $\kappa$ B-binding sites that are crucial for the TNF- $\alpha$  inducibility of this gene (20). To further test the involvement of the TAF<sub>II</sub>105-TFIID complex in A20 transcription we determined the effect of TAF<sub>II</sub>105 on the promoter activity of the A20 gene. 293 cells were transfected with a luciferase reporter gene under the control of the A20 promoter together with p65 subunit of NF- $\kappa$ B and TAF<sub>II</sub>105. TAF<sub>II</sub>105 enhances both, basal and p65 induced activity of the A20 promoter (Fig. 6A, columns 3 and 7), and this induction requires the presence of NF- $\kappa$ B proteins as TAF<sub>II</sub>105 fails to induce the A20 promoter activity in the presence of the NF- $\kappa$ B inhibitor protein, I $\kappa$ B (columns 5 and 9) or when NF- $\kappa$ B sites were deleted from the promoter (columns 11–14). We also examined the effect of the dominant negative mutant of TAF<sub>II</sub>105 on the A20 promoter activity. TAF<sub>II</sub>105 $\Delta$ C inhibited the basal and p65 induced activity of the A20 promoter (compare columns 1 and 4 to 6 and 10). The inhibition of basal and induced A20 promoter activity by TAF<sub>II</sub>105 $\Delta$ C is similar to that of I $\kappa$ B (columns 5 and 8).

We then tested the effect of TAF<sub>II</sub>105 on the TNF- $\alpha$  induced activity of the A20 promoter. Here again TAF<sub>II</sub>105 enhances the TNF- $\alpha$  induced promoter activity (Fig. 6B, column 3) and TAF<sub>II</sub>105 $\Delta$ C abolished this induction (column 6). In the presence of I $\kappa$ B, or when the NF- $\kappa$ B-binding site is deleted, TAF<sub>II</sub>105 fails to enhance A20 promoter activity (column 5) indicating that enhancement of TNF- $\alpha$  stimulated promoter activity by TAF<sub>II</sub>105 requires the NF- $\kappa$ B proteins. Similarly, TAF<sub>II</sub>105 further stimulated the activity of the A20 promoter

in the human fibroblast cell line HT1080 (Fig. 3C) indicating that the effect of TAF<sub>II</sub>105 on the A20 promoter is not restricted to 293 cells.

**A20 Suppresses TAF<sub>II</sub>105 $\Delta$ C-induced Cell Death More Efficiently than I $\kappa$ B $\alpha$ -induced Cell Death**—The early TNF- $\alpha$  induced gene A20 is a zinc finger-containing protein involved in modulation of TNF- $\alpha$  signaling (21) and inhibition of apoptotic proteases (22). A20 confers resistance to TNF- $\alpha$  cytotoxicity and to other apoptotic signals in certain cell types (15, 23–25). Since promotion of cell death by TAF<sub>II</sub>105 $\Delta$ C and I $\kappa$ B $\alpha$  is associated with inhibition of anti-apoptotic genes such as A20, we tested whether overexpression of the A20 gene would protect cells from the apoptotic effect of TAF<sub>II</sub>105 $\Delta$ C or I $\kappa$ B $\alpha$ . Cells were transfected with expression plasmids of TAF<sub>II</sub>105 $\Delta$ C or superdominant I $\kappa$ B $\alpha$  along with the green fluorescent protein plasmid and viability of the transfected cells was measured 48 h after addition of TNF- $\alpha$ . Similar marked reductions in cell survival were observed in cells expressing TAF<sub>II</sub>105 $\Delta$ C or superdominant I $\kappa$ B $\alpha$ , in response to TNF- $\alpha$  (Fig. 7A, columns 4 and 6, and B, middle panel). However, when the A20 gene was co-transfected with TAF<sub>II</sub>105 $\Delta$ C, the TNF- $\alpha$ -induced apoptosis was overcome and cell survival was significantly increased (Fig. 7A, compare columns 4 and 8, and B). It was noted that the A20 gene only partially blocked the TNF- $\alpha$ -induced apoptosis in cells expressing superdominant I $\kappa$ B $\alpha$  (compare columns 6 to 10). This suggests that full rescue from I $\kappa$ B $\alpha$ -mediated apoptosis requires expression of some other anti-apoptotic genes besides A20. Furthermore, the differential protective effect of A20 indicates that the inhibitory effect of TAF<sub>II</sub>105 $\Delta$ C on NF- $\kappa$ B anti-apoptotic genes is more restricted than that of



**FIG. 7. Effect of A20 expression on survival of TNF- $\alpha$ -induced cells.** **A**, human 293T cells were co-transfected with expression plasmids as indicated in the bottom of each column, and the GFP expression plasmid. Twenty-four hours after transfection TNF- $\alpha$  was added and 48 h later green fluorescent cells from three fields were counted. These results are average of four independent transfection experiments. **B**, representative pictures of TAF<sub>II</sub>105 $\Delta$ C transfected 293 cells rescued from apoptosis by A20 expression.

I $\kappa$ B $\alpha$  which is known to be a general inhibitor of NF- $\kappa$ B. Thus, it can be concluded that TAF<sub>II</sub>105 mediates activation of only a subset of NF- $\kappa$ B-dependent genes in response to TNF- $\alpha$ . This notion is consistent with the selective binding of TAF<sub>II</sub>105 by just one of the NF- $\kappa$ B complexes induced by TNF- $\alpha$ .

#### DISCUSSION

In this study we have dissected the molecular mechanism involved in activation of anti-apoptotic genes by TAF<sub>II</sub>105 and p65/RelA. Using biochemical and functional analyses of TAF<sub>II</sub>105-NF- $\kappa$ B interaction together with the characterization of a gene regulated by TAF<sub>II</sub>105 and NF- $\kappa$ B, we show that TAF<sub>II</sub>105 functions in a selective manner with respect to NF- $\kappa$ B. TAF<sub>II</sub>105 exhibits high affinity to NF- $\kappa$ B dimers composed of p65/p65 and p65/c-Rel subunits; interaction with heterodimer composed of p65/50, however, is significantly less efficient. Since in the cell the amount of TAF<sub>II</sub>105-TFIID complex is limited (26), it is likely that it will be targeted only by those NF- $\kappa$ B complexes for which it displays high affinity. Genes activated by the p65/p50 complex are, therefore, less likely to be regulated by TAF<sub>II</sub>105.

Identification of A20 as a gene regulated by TAF<sub>II</sub>105, and examination of its anti-apoptotic activity provided some evidence that the relative affinity of TAF<sub>II</sub>105 toward different forms of NF- $\kappa$ B dimers contributes to differential gene activation by NF- $\kappa$ B. The results showed that the TNF- $\alpha$ -induced mRNA level of the endogenous A20 gene is enhanced by TAF<sub>II</sub>105 expression and reduced by both dominant negative mutants of TAF<sub>II</sub>105 and I $\kappa$ B $\alpha$ . Consistent with the cell death protective function of the A20 gene, its expression in 293 cells shields them against apoptosis induced by the mutant TAF<sub>II</sub>105 protein and TNF- $\alpha$ , suggesting that down-regulation of A20 by TAF<sub>II</sub>105 $\Delta$ C is associated with the cell death response. Since the A20 gene only partially suppresses apoptosis induced by superdominant I $\kappa$ B $\alpha$  and TNF- $\alpha$ , full protection

requires additional genes activated by NF- $\kappa$ B as reported elsewhere (17). Importantly, the differential effect of A20 on TAF<sub>II</sub>105 $\Delta$ C and I $\kappa$ B $\alpha$  indicates that the inhibitory effect of TAF<sub>II</sub>105 $\Delta$ C on NF- $\kappa$ B activity is not as general as is that of I $\kappa$ B $\alpha$ , and is directed to certain specific genes. These results are compatible with the selective association of TAF<sub>II</sub>105 with one of NF- $\kappa$ B complexes induced by TNF- $\alpha$  in 293 cells. Hence, it is conceivable that NF- $\kappa$ B can utilize alternative pathways for transcription activation. Such alternative pathways might be dependent on the structure and composition of the target gene promoter as well as on the existence of other coactivators for NF- $\kappa$ B proteins. This idea is consistent with recent studies showing that transcription activation by the p65/RelA subunit of NF- $\kappa$ B can be mediated by multiple and distinct coactivators (9–13). It has been suggested that different coactivator complexes may act sequentially during the transcription activation process (28). Our results also raise the possibility that different coactivator subunits may function in the context of different target genes, suggesting that activator-coactivator interaction is important for specifying gene activation. The suggested mechanism provides a partial explanation as to how NF- $\kappa$ B containing complex activates different arrays of genes in response to different extracellular signals.

The expression pattern of TAF<sub>II</sub>105 and A20 genes in normal tissues is similar. TAF<sub>II</sub>105 protein levels are relatively high in certain B cell lines (26) and in normal murine lymphoid organs such as spleen and thymus (data not shown). Similarly, high levels of the murine A20 mRNA were also found in lymphoid organs, including the thymus and spleen (27). Since NF- $\kappa$ B is constitutively active in many lymphoid cell types, it is possible that large amounts of NF- $\kappa$ B-TAF<sub>II</sub>105-TFIID complex in lymphoid cells are responsible for the significant expression of the A20 gene in these cells.

The role of TAFs in transcription is not entirely understood. *In vitro* transcription studies have clearly indicated that TAFs are essential activation domains mediators, as well as promoter selectivity factors (29, 30). On the other hand genetic analysis of some yeast TAFs indicated that transcription from many promoters is not affected by their inactivation (31, 32). Moreover, recent studies revealed a mechanism of transcription activation that is TAF-independent (33, 34) and that certain TAFs are subunits of additional multiprotein complexes (35–37). It is therefore apparent that identifying the genes regulated by TFIID subunits and the mechanism by which these TAFs function *in vivo* are critical to understanding the role of TAFs. The previous finding that TAF<sub>II</sub>105 is an activation domain-specific coactivator of p65/RelA required for transcription activation of anti-apoptotic genes (9) together with the present characterization of TAF<sub>II</sub>105 as coactivator of a specific set of NF- $\kappa$ B genes, is an important step toward understanding how TAFs work and what role they play in specific gene regulatory pathways.

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#### REFERENCES

- Baldwin, A. S., Jr. (1996) *Annu. Rev. Immunol.* 14, 649–683
- Van Antwerp, D. J., Martin, S. J., Verma, I. M., and Green, D. R. (1998) *Trends Cell Biol.* 8, 107–111
- Mayo, M. W., Wang, C.-Y., Cogswell, P. C., Rogers-Graham, K. S., Lowe, S. W., Der, C. J., and Baldwin, A. S., Jr. (1997) *Science* 278, 1812–1815
- Reuther, J. Y., Reuther, G. W., Cortez, D., Pendergast, A. M., and Baldwin, A. S., Jr. (1998) *Genes Dev.* 12, 968–981
- Wang, C.-Y., Mayo, M. W., and Baldwin, A. S., Jr. (1996) *Science* 274, 784–787
- Sonenshein, G. E. (1997) *Semin. Cancer Biol.* 8, 113–119
- Foo, S. Y., and Nolan, G. P. (1999) *Trends Genet.* 15, 229–235
- Verma, I. M., Stevenson, J. K., Schwarz, E. M., Van Antwerp, D., and Miyamoto, S. (1995) *Genes Dev.* 9, 2723–2735
- Yamit-Hezi, A., and Dikstein, R. (1998) *EMBO J.* 17, 5161–5169
- Perkins, N. D., Felzien, L. K., Betts, J. C., Leung, K., Beach, D. H., and Nabel, G. J. (1997) *Science* 275, 523–527

11. Gerritsen, M. E., Williams, A. J., Neish, A. S., Moore, S., Shi, Y., and Collins, T. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 2927-2932
12. Guermah, M., Malik, S., and Roeder, R. G. (1998) *Mol. Cell. Biol.* **18**, 3234-3244
13. Naar, A. M., Beaurang, P. A., Zhou, S., Abraham, S., Solomon, W., and Tjian, R. (1999) *Nature* **398**, 828-832
14. Wong, G. H., Elwell, J. H., Oberley, L. W., and Goeddel, D. V. (1989) *Cell* **58**, 923-931
15. Opiari, A. W., Hu, J. R., Yabkowitz, R., and Dixit, V. M. (1992) *J. Biol. Chem.* **267**, 12424-12427
16. Chu, Z.-L., McKinsey, A. T., Liu, L., Gentry, J. J., Malim, M. H., and Ballard, D. W. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 10057-10062
17. Wang, C. Y., Mayo, M. W., Korneluk, R. G., Goeddel, D. V., and Baldwin, A. S., Jr. (1998) *Science* **281**, 1680-1683
18. Karsan, A., Yee, E., and Harlan, J. M. (1996) *J. Biol. Chem.* **271**, 27201-27204
19. Wu, M. X., Ao, Z., Prasad, K. V. S., Wu, R., and Schlossman, S. F. (1998) *Science* **281**, 998-1001
20. Krikos, A., Laherty, C. D., and Dixit, V. M. (1992) *J. Biol. Chem.* **267**, 17971-17976
21. Song, H. Y., Rothe, M., and Goeddel, D. V. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 6721-6725
22. Wissing, D., Mouritzen, H., and Jaattela, M. (1998) *Free Radic. Biol. Med.* **25**, 57-65
23. Jaattela, M., Mouritzen, H., Eliing, F., and Bastholm, L. (1996) *J. Immunol.* **156**, 1166-1173
24. Sarma, V., Lin, Z., Clark, L., Rust, B. M., Tewari, M., Noelle, R. J., and Dixit, V. M. (1995) *J. Biol. Chem.* **270**, 12343-12346
25. Fries, K. L., Miller, W. E., and Raab-Traub, N. (1996) *J. Virol.* **70**, 8653-8659
26. Dikstein, R., Zhou, S., and Tjian, R. (1996) *Cell* **87**, 137-146
27. Tewari, M., Wolf, F. W., Seldin, M. F., O'Shea, K. S., Dixit, V. M., and Turka, L. A. (1995) *J. Immunol.* **154**, 1699-1706
28. Cosma, M. P., Tanaka, T., and Nasmyth, K. (1999) *Cell* **97**, 299-311
29. Verrijzer, C. P., and Tjian, R. (1996) *Trends Biochem. Sci.* **21**, 338-342
30. Hoffmann, A., Oelgeschlager, T., and Roeder, R. G. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 8928-8935
31. Moqtaderi, Z., Bai, Y., Poon, D., Weil, P. A., and Struhl, K. (1996) *Nature* **383**, 188-191
32. Walker, S. S., Reese, J. C., Apone, L. M., and Green, M. (1996) *Nature* **383**, 185-188
33. Koleske, A. J., and Young, R. A. (1995) *Nature* **368**, 466-469
34. Oelgeschlager, T., Tao, Y., Kang, Y. K., and Roeder, R. G. (1998) *Mol. Cell* **1**, 25-31
35. Grant, P. A., Schieltz, D., Pray-Grant, M. G., Steger, D. J., Reese, J. C., Yates, J. R., and Workman, J. L. (1998) *Cell* **94**, 45-53
36. Ogryzko, V. V., Kotani, T., Zhang, X., Schlitz, R. L., Howard, T., Yang, X. J., Howard, B. H., Qin, J., and Nakatani, Y. (1998) *Cell* **94**, 35-44
37. Wiczorek, E., Brand, M., Jacq, X., and Tora, L. (1998) *Nature* **393**, 187-191
38. Schreiber, E., Matthias, P., Muller, M. M., and Schaffner, W. (1989) *Nucleic Acids Res.* **17**, 6419